INTERNATIONAL JOURNAL OF HYDROGEN ENERGY 40 (2015) 6319–6328

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Two-stage thermophilic fermentation and mesophilic methanogen process for biohythane production from palm oil mill effluent

Chonticha Mamimin [a], *Apinya Singkhala* [a], *Prawit Kongjan* [b,c], *Benjaporn Suraraksa* [d], *Poonsuk Prasertsan* [e], *Tsuyoshi Imai* [f], *Sompong O-Thong* [a,g,*]

[a] *Biotechnology Program, Department of Biology, Faculty of Science, Thaksin University, Phatthalung, 93210, Thailand*

[b] *Chemistry Division, Department of Science, Faculty of Science and Technology, Prince of Songkla University, Muang, Pattani, 94000, Thailand*

[c] *Bio-Mass Conversion to Energy and Chemicals (Bio-MEC) Research Unit, Department of Science, Faculty of Science and Technology, Prince of Songkla University, Muang, Pattani, 94000, Thailand*

[d] *National Center of Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathumthani, 12120, Thailand*

[e] *Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkhla, 90112, Thailand*

[f] *Division of Environmental Science and Engineering, Graduate School of Science and Engineering, Yamaguchi University, Yamaguchi, Japan*

[g] *Microbial Resource Management Research Unit, Faculty of Science, Thaksin University, Phatthalung, 93210, Thailand*

ARTICLE INFO

Article history:
Received 17 October 2014
Received in revised form
8 March 2015
Accepted 14 March 2015
Available online 7 April 2015

Keywords:
Thermophilic dark fermentation
Palm oil mill effluent
Two-stage process
Hydrogen and methane production
Biohythane
Microbial community

ABSTRACT

A two-stage thermophilic fermentation and mesophilic methanogenic process for biohythane production from palm oil mill effluent (POME) was investigated. The hydrogen and methane potential from POME were 170–200 L H_2 $kgCOD^{-1}$ and 210–292 L CH_4 $kgCOD^{-1}$, respectively. A continuous two-stage process with hydraulic retention time (HRT) of 2 d for hydrogen reactor and 15 d for methane reactor, obtained 34% higher energy yield than single stage methane production. The hydrogen and methane yields from two-stage were 210 L H_2 $kgCOD^{-1}$ and 315 L CH_4 $kgCOD^{-1}$, respectively with total energy yield of 15.34 MJ $kgCOD^{-1}$. Mixed hydrogen and methane (biohythane) production rate was 4.4 L biogas $L^{-1}d^{-1}$ with containing of 51% CH_4, 14% H_2 and 35% CO_2. Hydrogen reactor was dominated with hydrogen producing bacteria of *Thermoanaerobacterium thermosaccharolyticum*, while acetoclastic *Methanoculleus* sp. was the dominant methanogen in methane reactor. Two-stage process for biohythane production could efficiently for energy recovery from POME.

* *Corresponding author*. Biotechnology Program, Department of Biology, Faculty of Science, Thaksin University, Phatthalung, 93210, Thailand. Fax: +66 74693992.
E-mail address: sompong.o@gmail.com (S. O-Thong).

http://dx.doi.org/10.1016/j.ijhydene.2015.03.068

Introduction

Hydrogen is a promising energy carrier in the future. Hydrogen is high energy density, higher conversion efficiency of usable power and low generation of pollutants [1]. Microbial hydrogen fermentation from organic waste has been attracting attention as an environmental friendly process and high production rate with organic waste under realistic conditions which approaching practical levels [2]. Various organic materials such as cellulose and starch containing waste and food industrial wastewaters, cheese whey, olive mill wastewater and oil palm mill effluent can utilized for hydrogen gas production [3]. Hydrogen yield from microbial fermentation of organic waste such as apple processing wastewater, potato processing wastewater, food waste, starch wastewater and palm oil mill effluent was 92 [4], 128 [5], 57 [6], 92 [7] and 115 [8] ml H_2 $gCOD^{-1}$. Palm oil mill effluent (POME) is a suitable substrate for hydrogen production in term of hydrogen yield and large volume of POME in Thailand, Malaysia and Indonesia. Every one tonne of palm oil generated approximately 5–7.5 tonnes of POME. POME has high temperature (80–90 °C). Thermophilic process could be applied to POME with eliminating the need of cooling systems [9].

Successful hydrogen production from POME by microbial fermentation was achieved under thermophilic condition. Prasertsan et al. [10] obtained the continuous hydrogen production rate (HPR) of 9.1 l H_2 1^{-1} d^{-1} (16.9 mmol H_2 l^{-1} h^{-1}) by *Thermoanaerobacterium*-rich sludge under thermophilic condition (60 °C) at optimal values of 2 days HRT with an OLR of 60 gCOD l^{-1} d^{-1} in anaerobic sequencing batch reactor (ASBR) reactor. High temperatures are interesting approach to convert POME into hydrogen due to more favorable thermodynamics condition, less variety of fermentation end-products, low hydrogen partial pressure inhibition. However, a number of challenges must be overcome before hydrogen from POME can become economically feasible especially low substrate conversion efficiency. In a conventional microbial fermentation process only about 7.5–15% of the energy contained in organic waste is converted to hydrogen, the rest of the energy is contained in volatile fatty acids (VFA) [1]. 65% of the energy contained in the organic waste still remains in the liquid as VFA mainly butyric acid, acetic acid, lactic acid and propionic acid. Consequently, VFA could be converted into a suitable product or energy carrier such as methane via methanogenesis by many methanogens under anaerobic digestion [11]. In addition, it has been shown to be an energy efficient strategy for the conversion of a mixture of hydrogen and methane, known as hythane or biohythane [12] and can be used as an energy carrier in gas combustion engines.

Biohythane can be realized through a two-stage microbial fermentation, in the first stage substrate is fermented to hydrogen and VFA and VFA is conversed to methane in second stage [11]. The pH of 5–6 and optimal HRT of 1–3 days are optimized for acidogenic bacteria that could convert carbohydrate to hydrogen via the acetate and butyrate pathways. In the second stage, the remaining organic acids in the hydrogen reactor effluent is anaerobically converted to methane by methanogens under a neutral pH range of 7–8 and HRT of 10–15 days [13]. The two-stage could increased degradation efficiency and improved negative impacts of inhibitive compounds in feedstock with high yield and purity of products [14]. In addition, the two-stage process has advantages of increased the net energy balance, increased stability with better control of acid phase, higher organic loading rates, increased specific activity of methanogens leading to an increase in methane production rates and increased overall COD and VS reduction efficiencies compared to one-stage hydrogen or methane fermentation. The two-stage process also characterized by a significantly reduced fermentation time [11]. The two-stage hydrogen and methane process is based on the differences of physiology, growth kinetics, and sensitivity to environmental conditions between acidogens and methanogens. These two different groups are enriched separately in two tanks enabling optimized growth by maintaining proper environmental conditions in each reactor [15]. The thermophilic anaerobic process is reported to have an acceleration effect on the biochemical reactions and a higher efficiency in the degradation of organic matter in comparison to the mesophilic process. Higher methane production rates were observed under thermophilic conditions compared to mesophilic conditions [16]. However, Kim et al. [17] reported that thermophilic anaerobic digestion has a lack of process stability caused by high propionate threshold concentrations. Although the study and practice of individual hydrogen and methane production are widespread, there were only a few reports on the two-stage process of their co-production and no report on hydrogen and methane production from POME in two-stage thermophilic fermentation and mesophilic methanogenic process.

This study aims to develop a two-stage thermophilic fermentation and mesophilic methanogenic process for hydrogen and methane production from POME. The factors affecting on process stability and microbial community of continuous two-stage hydrogen and methane production from POME in ASBR reactors and mesophilic anaerobic digestion in up-flow anaerobic sludge blanket (UASB) reactors were investigated.

Materials and methods

Inoculum preparation and POME characteristics

Seed sludge for hydrogen production was collected from a palm oil mill biogas plant. The sludge was enriched for thermophilic hydrogen producing bacteria according to Mamimin et al. [18]. The enriched sludge has a volatile suspended solids (VSS) concentration of 6.0 g l^{-1} was gradually acclimatized with POME by successive transfer to increasing concentration of POME from 50% to 100%. The acclimatized sludge was operated by discharged of 50% cultured broth and addition of 50% fresh POME every 24 h for 10 times. Then, the sludge was used in batch test and seeded into an ASBR reactor for continuous operation. An anaerobic digester sludge obtained from a palm oil mill biogas plant was used as a seed sludge for biomethane potential and mesophilic methanogenic reactor without treatment. Anaerobic digester sludge was placed in an incubator for 5 days until the biogas production was stopped in order to minimize the contribution of organic

materials contained in the inoculum. The inoculums was contained of 15.4 g l^{-1} total solids (TS), 13.6 g l^{-1} volatile solids (VS), and 11.2 g l^{-1} volatile suspended solids (VSS). POME from the receiving tank of Trang Palm Oil Co., Ltd. in southern Thailand had the following characteristics: high temperature (90 °C), pH 4.7, brownish color, 10.6% oil content, 14.2 g total carbohydrate l^{-1}, 4.2% total solids, 0.8% suspended solids, 3.3% volatile solids, 85.5 gCOD l^{-1}, 830 mg total Kjeldahl nitrogen l^{-1}, 130 mg total phosphorus l^{-1} and 3 mg iron l^{-1}. Table 1 shows the characteristics of the POME. Raw POME was supplemented with iron, nitrogen and phosphorus at concentration of 257 mg Fe^{2+} l^{-1}, C/N ratio of 95 adjusted by peptone as nitrogen source, and C/P ratio of 550 adjusted using $Na_2HPO_4 \cdot 2H_2O$ and the pH was adjusted to 5.5 by $NaHCO_3$ before being fed to the ASBR reactor [19].

Biochemical hydrogen potential (BHP) and biomethane potential (BMP)

The BHP and BMP of POME were identified in batch assays under thermophilic conditions and mesophilic conditions, as described previously by Giordano et al. [20]. The experiments were carried out in batch conditions, using 500 ml glass serum bottles. POME was tested at different initial COD loading levels of 20, 40 and 60 gCOD l^{-1}. Additionally, for testing the inoculum quality, positive controls with 20 g l^{-1} of cellulose and 20 g l^{-1} of glucose instead of samples were also included for observation. BHP test reactors, POME was mixed with 40 ml of inoculums and adjusted volume to 200 ml by water. Reactors were manually mixed every day and maintained at static conditions and constant temperature (55 °C) in a thermostatic cabinet for 5 days. When the biological hydrogen production ceased, the reactors were opened and another 120 g of anaerobic digestion sludge were introduced into the reactors and incubated at mesophilic condition for 45 days in order to evaluate the CH_4 production. The reactors were manually mixed every day during the first 7 days and every 2 days for the rest of the experimental duration and then maintained at static conditions. Biogas production was determined through the use of the water replacement method [21]. Biogas composition in the headspace of the vials was monitored by GC-TCD according to Hniman et al. [22]. The gas produced by the negative control bottles with inoculum was subtracted from the actual gas produced of each treatment.

Table 1 – Chemical characteristics of palm oil mill effluent used in this study.

Parameter	Concentration (g l^{-1})
Biochemical oxygen demand (BOD)	48.3 ± 0.6
Chemical oxygen demand (COD)	85.5 ± 0.3
Total carbohydrate	16.2 ± 0.2
Total nitrogen	0.83 ± 0.1
Ammonium–nitrogen	0.03 ± 0.001
Total phosphorus	0.13 ± 0.001
Phosphate	0.021 ± 0.001
Oil	10.6 ± 0.02
Total solids (TS)	62.0 ± 0.3
Volatile solids (VS)	55.0 ± 0.4
Suspended solids (SS)	8.5 ± 0.2
Ash	4.2 ± 0.2
Fe	0.003 ± 0.0001

Reactor operations and monitoring

Two-stage thermophilic fermentation and mesophilic methanogenic process was compared with single stage methane operation. Fig. 1 illustrates the schematic diagram of the experimental apparatus used in this study. The apparatus was composed of three parts including mixing tank, ASBR reactor for hydrogen production and UASB reactor for methane production. The first part is a mixing for making uniformly high solid slurry. The well-mixed fluid slurry in the mixing tank was fed to the ASBR, which was a thermophilic hydrogen production reactor with an effective volume of 200 ml. POME was intermittent mixed feed into the reactor with flow rate of 100 ml d^{-1} with 24 h cycles. Each cycle consisted of 30 min fill, 22 h 40 min reaction, 30 min settlement, 10 min draw and 10 min idle. ASBR was operated at 55 °C, pH of 5.5 and 2-d HRT. The 100 ml of seed inoculum were added to the hydrogen production reactors and the other half was filled with the POME. The operation reached steady state when hydrogen gas content, biogas volume and pH were stable (less than 10% variation). The third column was a mesophilic methanogenic reactor (UASB) with an effective volume of 3 l. A gas meter was installed in each reactor to record gas amount automatically. The seed inoculum were added to the methane production reactors to fill the 80% of the effective volume, and the other 20% was filled with effluent from hydrogen production reactor. The atmosphere of the methane reactor was exchanged by nitrogen gas. Second stage was operated at room temperature 28–34 °C, pH of 7.5 and 20, 15 and 10-d HRT. After 1–2 days, gas production was confined from the reactor, rather than continuously fed into the reactor was initially. In order to prevent the volatile fatty acids (VFAs) accumulation in the methanogenic reactor at short HRT of 20 days were operated

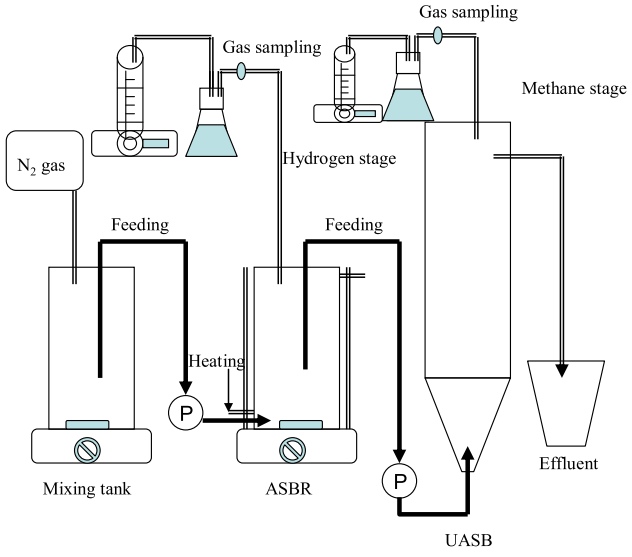

Fig. 1 – Schematic diagram of two-stage hydrogen and methane experimental apparatus.

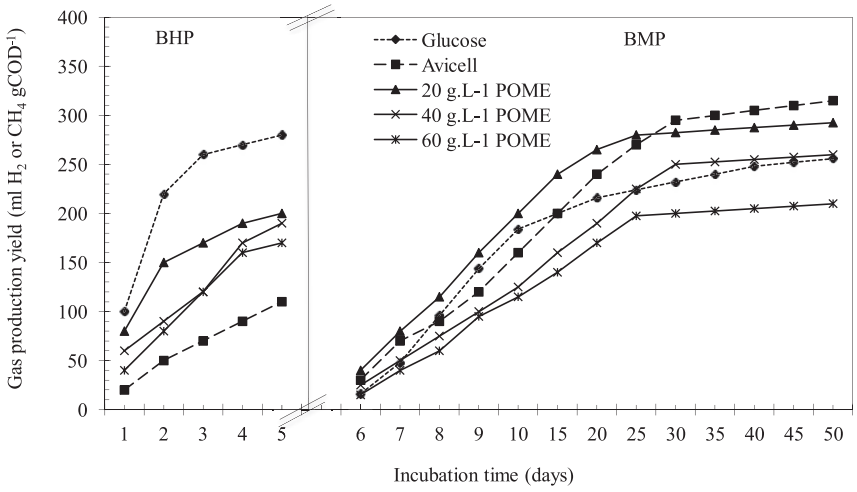

Fig. 2 – Cumulative gas (H_2 and CH_4) production during the two-stage biochemical hydrogen potential (BHP) and biochemical methane potential (BMP) tests of POME at difference initial organic loading.

at the first month. The HRTs of methane production reactor were vary from 10 to 20 d and pH controlled between 7.3 and 7.5. Second stage was tested at three different distributions of HRT between hydrogen and methane reactors of 2:20, 2:15 and 2:10 d, respectively. The two-stage ASBR and UASB were routinely monitored for the pH, gas production and composition, volatile fatty acid (VFA) distributions, chemical oxygen demand (COD) and suspended solids (SS). The steady-state condition (pH over 7.3 and VFA less than 1000 mg l^{-1}) was confined from the methanogenic reactor. Change of distributions of HRT between hydrogen and methane reactors was done after 2-week steady state conditions. The single stage UASB was run as a control for methane production with the HRT of 17 days at 35 °C, pH of 7.3–7.5.

Microbial communities analysis

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was used to study microbial community structure in the hydrogen production stage and methane production stage as pervious described by Kongjan et al. [12]. Most of the bands were excised from the gel and re-amplified. After re-amplification, PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Closest matches for partial 16S rRNA gene sequences were identified by database searches in Gene Bank using BLAST [23].

Analytical methods

Biogas production performance was monitored daily by determining the H_2, CO_2, H_2S and CH_4 concentrations by GC-TCD (Hewlett Packard, HP 6850) as previously described by O-Thong et al. [7]. The gas samples (100 μl for methane and 500 μl for hydrogen) were injected in duplicate. Volatile fatty acids (VFA) were analyzed by gas chromatography as previously described by Hniman et al. [22]. Chemical oxygen demand (COD), pH, suspended solid (SS), oil concentration, total phosphorus and total Kjeldahl nitrogen (TKN) were determined in accordance with the procedures described in the Standard Methods [24]. Ammonium-nitrogen and phosphate concentrations were analyzed using commercial test kits from Spectroquant (Merck Ltd., Germany). The total carbohydrate content was analyzed by the Anthrone method [25]. The hydrogen and methane yields obtained from each kilogram of COD in POME was calculated according to Zhu et al. [26]. The densities of hydrogen and methane gas used for the calculation were 0.09 mg ml^{-1} and 0.72 mg ml^{-1}. The heating values of the hydrogen and methane used were 142 kJ g^{-1} and 55.6 kJ g^{-1} [27]. Statistical significance was defined as $P < 0.05$ by t-test.

Results and discussion

Two-stage biohydrogen and biomethane potential of POME

In all biohydrogen potential (BHP) and biomethane potential (BMP) tests gas production started almost immediately indicating that a short lag phase. Glucose fermentation showed a hydrogen and methane recovery from initial COD was 18% and 80%, respectively indicating good inoculums. The H_2 concentration in the biogas of the BHP tests ranged between 45% and 60% with CO_2 as the remaining fraction and no CH_4 was detected. Gas production from POME reached an H_2 yield of 130–200 ml H_2/g COD after 2 days, which is within the range obtained in other studies [8] under thermophilic conditions. It is noteworthy that more than 90% of the H_2 was produced in approximately 2 days (Fig. 2). At the end of the BHP tests, acetic and n-butyric acids represented the main fermentative products reaching the concentration of 23450–3580 and 3250–4250 mg l^{-1} at all initial loading test (data not show). The VFAs production caused a significant pH decrease from 5.5 to 4.3 after 5 days of incubation. Methane production from hydrogenic effluent was yield of 210–292 ml CH_4 $gCOD^{-1}$. During the BMP test, the methane concentration in the biogas ranged between 55 and 64%. VFA from hydrogen production

process was completely degraded in BMP test with concentration below 30 mg L^{-1} of acid. Although methane production lasted approximately 50 days, about 90% of the total CH_4 was produced during the first 15–20 days.

Two-stage BHP and BMP of POME showed a hydrogen and methane recovery from initial COD was 11–13% and 60–83%, respectively (Table 2). The total gas recovery also demonstrated that much more energy could be gained by a two-stage hydrogen and methane production. 90% of methane production from POME hydrogenic effluent can achieved within 15–20 days. Results indicating that optimum HRT for hydrogen production in first stage and methane production in second stage should be 2 days and 15–20 days, respectively. The results agreed with Liu et al. [27] that reported HRT for first stage for hydrogen from organic fraction of municipal solid wastes are 1–2 days and HRT second stage for methane production are 10–15 days, respectively. The volume of reactor for methane production is about 10 times as large as that of hydrogen production and 2–4 times smaller than single stage methane production.

Continuous hydrogen production

The selected condition (OLR of 60 g COD l^{-1} d^{-1} and HRT 2 d) for hydrogen production from POME by thermophilic mixed culture using ASBR were established according to our previous study [10]. The hydrogen gas production rate observed from the ASBR reactor is show in Fig. 3. The maximum hydrogen production rate record from this reactor was 1.84 l H_2 l^{-1} d^{-1}, which was observed on day 90–120. The average rate over the entire experiment was 1.8 l H_2 l^{-1} d^{-1}. The hydrogen concentration in the biogas was 48–60% with an average concentration of 55%. The methane concentration was not observed in the biogas, indicating that a no methanogenic activity existed in the hydrogen reactor. No hydrogen sulfide was detected. The average and maximum hydrogen yields were calculated to be 210 ml H_2 $gCOD^{-1}$ respectively, with acetic acid and butyric acid as main products in fermentation soluble metabolites (Fig. 3.). The hydrogen produced from each liter of POME was 9.0 l H_2 l^{-1}POME. The hydrogen yield was higher than hydrogen yield (4.7 l H_2 l^{-1}POME) from POME in batch reactor under thermophilic condition [28]. The hydrogen yield was also higher than hydrogen yield (2.64 l H_2 l^{-1}POME) from POME in CSTR operating at HRT 4 days under thermophilic condition [29]. Thermophilic hydrogen fermentation is enhancing biogas production yields by reducing biogas solubility and lowering the H_2 partial pressure in the head space. Thermophilic hydrogen fermentation also improving the degradation efficiency at high temperatures [8]. Higher temperatures (60 °C) are energetically more favorable for biological hydrogen production, enabling thermophiles to reach higher hydrogen yields than mesophiles. Moreover, strictly anaerobic thermophilic conditions seem to restrict contamination by hydrogenotrophic methanogens [31].

The characteristics of the hydrogenic effluent are presented in Table 3. COD remaining was 59.5 g l^{-1} on average, resulting in a removal rate of 38%. The COD removal efficiency was low under hydrogen production stage, since mineralization of organic compounds under anaerobic conditions in not complete unless methane gas was produced. The decomposition of suspended solids (SS), which reflected solubilization of solid material during operation, was approximately 70% of SS in influent was decomposed in hydrogen production stage. Thermophilic fermentation process by ASBR reactor suitable for high suspended solid waste with higher hydrolysis activity, which is the bottleneck for degradation of high suspended solid waste like POME. ASBR has been used for hydrogen production due to a promising anaerobic high rate processes with short HRT and high degree of process flexibility operation [30] and also prevention of interference of high suspended solids in the feeding substrates [10]. Acetic acid and butyric acid were accumulated with a little production of ethanol, propanol, propionic acid and valeric acid in hydrogen production stage. The concentration of acetic acid, butyric acid, ethanol, propanol, propionic acid and valeric acid was 4866, 7855, 200, 408, 150 and 146 mg l^{-1} , respectively.

DGGE profiling of sludge from hydrogen reactor at steady state illustrated that *Thermoanaerobacterium* spp., such as *Thermoanaerobacterium thermosaccharolyticum* and *Thermoanaerobacterium acidoterolans* were dominant (Fig. 4a). The optimal growth of *T. thermosaccharolyticum* was in the pH range from 5 to 6 and the optimum temperature was 60 °C. *T. thermosaccharolyticum* was also reported as the dominant species in thermophilic hydrogen reactors from other study and played an important role in hydrogen production [31]. *Thermoanaerobacterium* can use complex carbohydrates such as starch, xylan, cellulose and simple sugars for hydrogen production. Different species of *Thermoanaerobacterium* are known for their capability to produce hydrogen, such as *T. thermosaccharolyticum*, *Thermoanaerobacterium polysaccharolyticum*, *Thermoanaerobacterium zeae*, *Thermoanaerobacterium lactoethylicum* and *Thermoanaerobacterium aotearoense* [31]. Lebuhn et al. [32] observed the predominance of *Thermoanaerobacterium* in biogas production from lignocellulose-rich substrates at 55 °C. In the present study, the specific activity of this group of microorganisms was also extremely high. Hydrogen yield, and growth rate of hydrogen producing bacteria increased as the thermophilic temperature thermophilic fermentation presents

Table 2 – Summary of hydrogen and methane recovery from POME.

Substrate	Initial COD loading ($gCOD\ l^{-1}$)	H_2 yield $L\ kg^{-1}$ COD	CH_4 yield $L\ kg^{-1}$ COD	H_2 recovery (%COD COD^{-1})	CH_4 recovery (%COD COD^{-1})	Total gas recovery (%COD COD^{-1})
Glucose	20	280	280	18.6	80	98.6
Avicell	20	110	315	6.6	90	96.6
POME	20	200	292	13.3	83.4	96.7
POME	40	190	260	12.6	74.3	86.9
POME	60	170	210	11.3	60	71.1

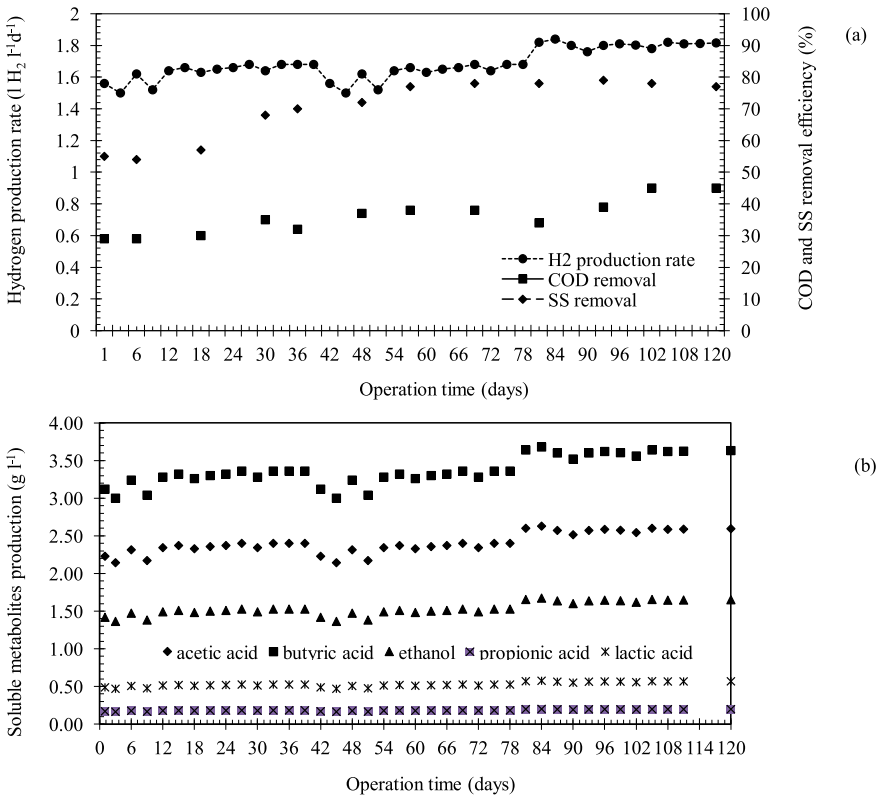

Fig. 3 – Profiles of hydrogen production rate, COD and SS removal efficiency (a) and soluble fermentation metabolites (b) from POME in ASBR operating at 2-d HRT under thermophilic condition.

a number of advantages in comparison to the mesophilic process, such as higher yields of hydrogen, more efficient degradation of organics, and more resistance to contamination [33,34].

Continuous methane production

A two-stage thermophilic-mesophilic anaerobic digestion was operated solely on POME. The first-stage thermophilic

Table 3 – Process performance and effluent characteristics on two-stage hydrogen and methane production and single stage methane production.

	Two stage process operation		Single stage
	Hydrogen reactor	Methane reactor	Methane reactor
Culture condition			
HRT (d)	2	15	17
LOR (gCOD l^{-1} d^{-1})	60	6	6
pH	5.5	7.3–7.5	7.3–7.5
Gas production (L biogas l^{-1} d^{-1})	1.84	2.4	1.72
Gas Yield (ml H_2 or CH_4 $gCOD^{-1}$)	210	315[a]	227[a]
Removal efficiency			
Total COD (%)	38	95	84
SS (%)	75	96[a]	73[a]
Carbohydrate consumption (%)	92	99	95
Effluent			
pH	4.3	8.2	7.9
Ethanol (mg l^{-1})	200	10	40
Propanol (mg l^{-1})	408	5	50
Acetic acid (mg l^{-1})	4866	20	150
Propionic acid (mg l^{-1})	150	0	50
Butyric acid (mg l^{-1})	7855	50	340
Valeric acid (mg l^{-1})	146	0	30

[a] Significant ($P < 0.05$).

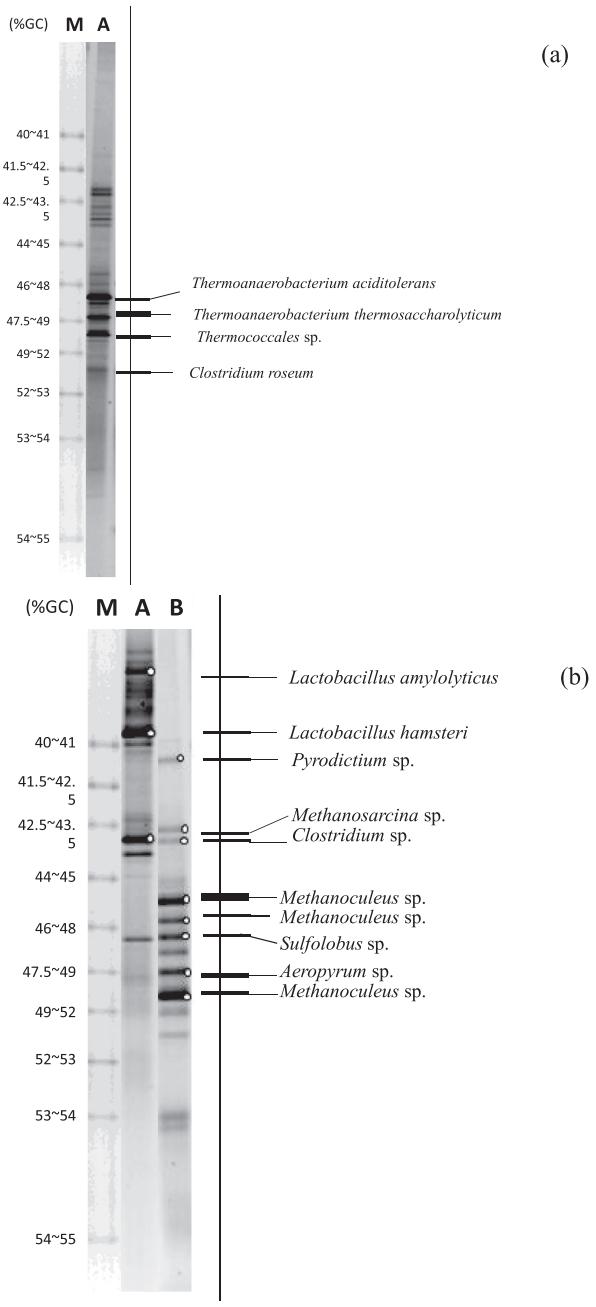

Fig. 4 – DGGE profile of bacterial community in sludge (a) from ASBR reactor operated for hydrogen production from POME under thermophilic condition and (b) from second stage UASB reactor operated for methane production from hydrogenic effluent under mesophilic condition (M) DGGE marker (A) bacterial profile and (B) archaea profile.

reactor (HRT 2 days) was operated at 55 °C. The second-stage mesophilic digester (HRT 15 days) was held at a room temperature of 28–34 °C. For comparison with a single-stage mesophilic process, the mesophilic digester was also operated separately with an HRT of 17 days and temperature of 28–34 °C. Methane gas production from effluent of hydrogen stage was examined using UASB. The hydraulic retention time (HRT) was gradually increased step by step after steady state at applied HRT was established. The gas production rate observed from the methane reactor is shown in Fig. 5. During start-up with 20 d HRT, methane was produced continuously. There was no delay of methane production observed in the reactor during start-up stage, demonstrating that the effluent of hydrogen reactor was readily usable for methanogenesis. The methane production declined after increased HRT to 10 d at day of 77 and then recover by 15 d HRT, indicating that 15 d HRT is the maximum HRT for methane production. During steady state, biogas productions were stable and COD removal greater than 90% was observed at HRT 15 days. The average methane production rate observed in the steady state of HRT 20, 15 and 10 d were 1.8, 2.53 and 1.2 l CH_4 l^{-1} d^{-1}. The maximum methane production rate of 2.6 l CH_4 l^{-1} d^{-1} was achieved at HRT 15 days and LOR of 6 gCOD l^{-1} d^{-1}. The methane concentration in the gas produced was 70–76% (v/v) with an average of 73%. The methane yield based on COD basis in the reactor was 315 ml CH_4 $gCOD^{-1}$. Two-stage hydrogen and methane enhanced degradation of organic waste and favors a high product yields and biogas quality. This methane yield of 315 ml CH_4 $gCOD^{-1}$ was higher than two stage methane production from POME (220 ml CH_4 g COD^{-1}) that observed by Chaisri [35]. It was the same methane yield (320 ml CH_4 g COD^{-1}) with single stage methane production from POME in high rate fixed film bioreactor observed by Najafpour [36]. The pH was stable at 7.3–7.5 throughout either experiment without pH regulation in two-stage. These results clearly indicate that two-stage reduce retention time of methanogenic process and increase methane production rate.

The characteristics of the effluent of the methane reactor are presented in Table 3. In the methane effluent from two-stage has COD removal efficiency more than 90% and SS removal efficiency more than 90% at optimum condition (HRT 15). While methane effluent of single stage has 84% and SS removal efficiency more than 73% at HRT of 17 d. Result indicating that incomplete degradation occurred in single stage at the same HRT result in lower gas production. One stage methane production has problematic when dealing with complex wastewater that contains recalcitrant. These compounds are generally inhibitive to biodegradation by microorganisms and thus cause low bioconversion efficiency [37]. Roberts et al. (1999) demonstrated that two-stage thermophilic and mesophilic system can be operated with much lower retention times and still achieve good VS reduction.

DGGE profiling of sludge from two-stage UASB reactor illustrated that *Clostridium* sp., *Lactobacillus hamster*, and *Lactobacillus* sp. were dominant bacteria in methane production stage. *Methanosarcina* sp. and *Methanoculleus* sp. were dominant and played an important role in methane production (Fig. 4b). The dominant archaea was *Methanosarcina* sp., which belonged to acetoclastic methanogens [38]. *Methanosarcina* species were reported to be dominant at high acetate concentration (>1.2 mM), and the results were consistent with the high acetate concentrations in all the hydrogen effluent that feed to methane reactors (Table 3). Others dominant archaea bands were related to *Methanoculleus* species, which were responsible for hydrogenotrophic

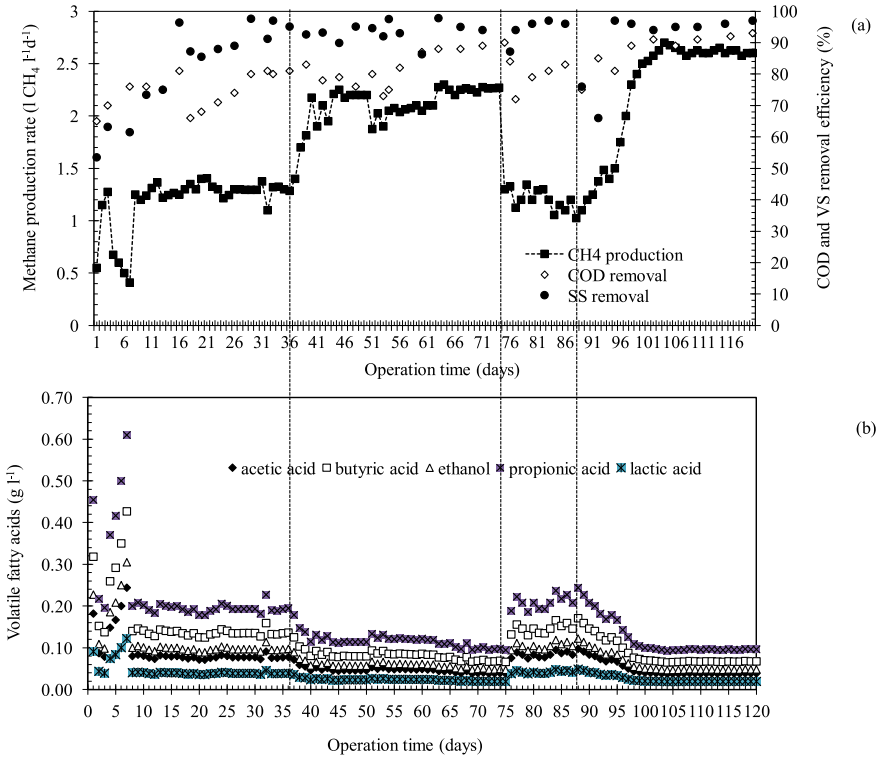

Fig. 5 – Profiles of continuous methane production rate, COD and SS removal efficiency (a) and volatile fatty acids concentration (b) in second stage using up-flow anaerobic sludge blanket reactor at various HRT.

methanogenesis [39]. The methanogenic performance on the two-stage process operation was high as that from single stage operation, suggesting that hydrogen fermentation could be efficiently coupled with a subsequent step for methanogenic process.

Total energy yield of two-stage hydrogen and methane production process

The hydrogen production stage was operated in ASBR with maximum hydrogen production rate of 1.84 l H_2 l^{-1} d^{-1}. The

Table 4 – Energy yield from single stage hydrogen production, single stage methane production and two-stage hydrogen and methane production by mixed cultures.

Reactor	Feedstock	Temperature (°C)	Initial pH	Gas production rate (l gas $l^{-1}d^{-1}$)	Energy production rate (kW l-reactor^{-1})	Gas production yield (l gas kg COD^{-1})	Energy yield (MJ $kgCOD^{-1}$)	Reference
ACF	POME	37	5.0	0.45 (H_2)	0.0015	239	3.05	[43]
CSTR	POME	55	5.5	2.64 (H_2)	0.0093	214	2.73	[29]
ASBR	POME			2.44 (H_2)	0.0086	278	3.55	[8]
CSTR	Food waste	55	5.5	0.04 (H_2)	0.0001	273	3.49	[6]
UASB	Rice winery wastewater	55	5.5	0.15 (H_2)	0.0005	270	3.45	[46]
ASBR	POME	55	5.5	1.8 (H_2)	0.0063	210	2.72	This study
UFAF	POME	37	7.0	2.54 (CH_4)	0.0280	63	2.52	[35]
UASFF	POME	37	7.5	0.34 (CH_4)	0.0037	310	12.41	[36]
CSTR	POME	35	7.5	1.73 (CH_4)	0.0190	227	9.08	This study
CSTR and CSTR	Olive pulp	35	5.5	0.15 (H_2)	0.0005	144	1.84	[44]
		35	7.5	1.13 (CH_4)	0.0125	98	3.90	
UASB and UASB	Desugared molasses	55	5.5	5.6 (H_2)	0.0197	66	0.84	[45]
		55	7.5	3.4 (CH_4)	0.0374	120	4.80	
CSTR and CSTR	Potato waste	37	5.5	1.2 (H_2)	0.0042	22	0.28	[27]
		37	7.5	0.51 (CH_4)	0.0056	250	10.02	
ASBR and UASB	POME	55	5.5	1.8 (H_2)	0.0063	210	2.72	This study
		35	7.5	2.6 (CH_4)	0.0286	315	12.62	

*ACF = Anaerobic contact filter, FFB = Fixed film bioreactor, UASSF = Up-flow anaerobic sludge-fixed film reactor, UFAF = Up-flow anaerobic filter reactor, CSTR = continuous stirred-tank reactor, ASBR = Anaerobic sequencing batch reactor, UASB = Up-flow anaerobic sludge blanket reactor.

methane stage was operated in UASB with maximum methane production rate were 2.6 l CH_4 l^{-1} d^{-1}. Biogas production from two-stage was 34% higher than one stage methane production and 90% higher than one stage hydrogen production. The overall removal efficiencies of COD and SS of two-stage were 10% increase when compared with one stage. Based on the hydrogen and methane yields observed in this research, the average energy yield and corresponding maximum value obtained from each kilogram of COD in POME was calculated according to Zhu et al. [26]. The hydrogen and methane yields from palm oil mill effluent were 210 l H_2 $kgCOD^{-1}$ and 315 l CH_4 $kgCOD^{-1}$, respectively with total energy yield of 15.34 MJ $kgCOD^{-1}$. The energy yield from POME with one and two-stage was shown in Table 4. The overall energy recovery from POME by two stage was also higher than one stage hydrogen production and one stage methane production from others report [26,40]. The total energy produced, approximately 13% of the energy was obtained from hydrogen production. In the perspective of the energy yield, the hydrogen fermentation alone was not competitive with two-stage hydrogen and methane production. More importantly, as demonstrated in this research, the hydrogen production could improve the methane production and overall digestion efficiency. Mixed hydrogen and methane (biohythane) production rate was 4.4 l biogas l^{-1} d^{-1} with biogas composting of 51% CH_4, 14% H_2 and 35% CO_2 was obtained by two-stage thermophilic fermentation and mesophilic methanogenic process. The mixed hydrogen and methane (biohythane) shown to be energy efficient strategies for the energy recovery from organic wastes [41,42].

Conclusions

A two-stage thermophilic fermentation and mesophilic methanogenic process shown very promising method for the combined energy recovery and removal of COD and SS in POME with the sequential anaerobic production of hydrogen and methane production. The hydrogen production stage was operated in anaerobic sequencing batch reactor (ASBR) under a pH of 5.5, temperature of 55 °C hydraulic retention time (HRT) of 2 d and organic loading rate (OLR) of 60 gCOD l^{-1} d^{-1} with maximum hydrogen production rate of 1.84 l H_2 l^{-1} d^{-1} and an average of 1.8 l H_2 $l^{-1}d^{-1}$. The maximum and average of methane production rate in the second stage were 2.6 and 2.4 l CH_4 l^{-1} d^{-1}, respectively at 15 d HRT. Mixed hydrogen and methane production rate was 4.4 l biogas l^{-1} d^{-1} with biogas composting of 51% CH_4, 14% H_2 and 35% CO_2. The hydrogen and methane yields from palm oil mill effluent were 210 l H_2 $kgCOD^{-1}$ and 315 l CH_4 $kgCOD^{-1}$, respectively. The sequential generation of hydrogen and methane from POME markedly increases the energy yield with 34% higher than single stage methane production and 90% higher than single stage hydrogen production.

Acknowledgement

The authors would like to thank the Research and Development Institute Thaksin University (RDITSU) RTA5780002, Agricultural Research Development Agency (ARDA), and the Thailand Research Fund, through grant number MRG5580074, for financial support.

REFERENCES

[1] Hallenbeck PC, Ghosh D. Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol 2009;27:287–97.
[2] Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Espinosa RD. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 2004;22(9):477–85.
[3] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzym Microb Technol 2006;38:569–82.
[4] Van Ginkel SW, Oh SE, Logan BE. Biohydrogen gas production from food processing and domestic wastewaters. Int J Hydrogen Energy 2005;30:1535–42.
[5] Pan J, Zhang R, El-Mashad HM, Sun H, Ying Y. Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. Int J Hydrogen Energy 2008;33:6968–75.
[6] Shin HS, Youn JH, Kim SH. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. Int J Hydrogen Energy 2004;29:1355–63.
[7] O-Thong S, Hniman A, Prasertsan P, Imai T. Biohydrogen production from cassava starch processing wastewater by thermophilic mixed cultures. Int J Hydrogen Energy 2011;36(5):3409–16.
[8] O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland NK. Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. Enzym Microb Technol 2007;41:583–90.
[9] Gobi K, Vadivelu VM. By-products of palm oil mill effluent treatment plant–a step towards sustainability. Renew Sustain Energy Rev 2013;28:788–803.
[10] Prasertsan P, O-Thong S, Birkeland NK. Optimization and microbial community analysis for production of biohydrogen from palm oil mill effluent by thermophilic fermentative process. Int J Hydrogen Energy 2009;34:7448–59.
[11] Liu Z, Zhang C, Lu Y, Wu X, Wang L, Wang L, et al. States and challenges for high-value biohythane production from waste biomass by dark fermentation technology. Bioresour Technol 2013;135:292–303.
[12] Kongjan P, O-Thong S, Angelidaki I. Performance and microbial community analysis of two-stage process with extreme thermophilic hydrogen and thermophilic methane production from hydrolysate in UASB reactors. Bioresour Technol 2011;102:4028–35.
[13] Lee D, Ebie Y, Xu K, Li Y, Inamori Y. Continuous hydrogen and methane production from high-solid food waste in the two stage thermophilic fermentation process with the recirculation of digester sludge. Bioresour Technol 2010;101:42–7.
[14] Li WW, Yu HQ. From wastewater to bioenergy and biochemicals via two-stage bioconversion processes: a future paradigm. Biotechnol Advan 2011;29:972–82.
[15] Demirel B, Yenigun O. Two-phase anaerobic digestion processes: a review. J Chem Technol Biotechnol 2002;77:743–55.
[16] Watts S, Hamilton G, Keller J. Two-stage thermophilic-mesophilic anaerobic digestion of waste activated sludge from a biological nutrient removal plant. Wat Sci Tech 2006;53(8):149–57.

[17] Kim M, Bae W, Spence R. Improved anaerobic process efficiency using mesophilic and thermophilic elutriated phased treatment. J Environ Eng 2001;130(9):960–6.
[18] Mamimin C, Thongdumyu P, Hniman A, Prasertsan P, Imai T, O-Thong S. Simultaneous thermophilic hydrogen production and phenol removal from palm oil mill effluent by *Thermoanaerobacterium*-rich sludge. Int J Hydrogen Energy 2012;37(20):15598–606.
[19] O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland NK. Optimization of simultaneous thermophilic fermentative hydrogen production and COD reduction from palm oil mill effluent by *Thermoanaerobacterium*-rich sludge. Int J Hydrogen Energy 2008;33:1221–31.
[20] Giordano A, Cantu C, Spagni A. Monitoring the biochemical hydrogen and methane potential of the two-stage dark-fermentative process. Bioresour Technol 2011;102(6):4474–9.
[21] Owen WF, Struckey DC, Healy JB, Mccarty PL. Bioassay for monitoring bio- chemical methane potential and anaerobic toxicity. Water Res 1979;13:485–92.
[22] Haniman A, O-Thong S, Prasertsan P. Developing a thermophilic hydrogen producing microbial consortia from geothermal spring for efficient utilization of xylose and glucose mixed substrates and oil palm trunk hydrolysate. Int J Hydrogen Energy 2011;36:8785–93.
[23] Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 1998;73(1):127–41.
[24] APHA, AWWA, WEF. Standard methods for the examination of water and wastewater. 21th ed. Washington DC: American Public Health Association; 1999.
[25] Morris DL. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science 1948;107:254–5.
[26] Zhu H, Stadnyk A, Beland M, Seto P. Co-production of hydrogen and methane from potato waste using a two-stage anaerobic digestion process. Bioresour Technol 2008;99:5078–84.
[27] Liu D, Liu D, Zeng RJ, Angelidaki I. Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Res 2006;40:2230–6.
[28] Atif AAY, Fakhru'l-Razi A, Ngan MA, Morimoto M, Iyuke SE, Veziroglu NT. Fed batch production of hydrogen from palm oil mill effluent using anaerobic microflora. Int J Hydrogen Energy 2005;30:1393–7.
[29] Ismail I, Hassan MA, Abdul Rahman N, Chen SS. Thermophilic biohydrogen production from palm oil mill effluent (POME) using suspended mixed culture. Biomass Bioenergy 2010;34:42–7.
[30] Kim SH, Han SK, Shin HS. Performance comparison of a continuous-flow stirred tank reactor and an anaerobic sequencing batch reactor for fermentative hydrogen production depending on substrate concentration. Wat Sci Technol 2005;52:23–9.
[31] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. Int J Hydrogen Energy 2008;33:1204–14.
[32] Lebuhn M, Hanreich A, Klocke M, Schlüter A, Bauer C, Pérez CM. Towards molecularbiomarkers for biogas production from lignocellulose-rich substrates. Anaerobe 2014;29:10–21.
[33] Zhang T, Liu H, Fang HHP. Biohydrogen production from starch in wastewater under thermophilic condition. J Environ Manag 2003;69(2):149–56.
[34] Mu Y, Wang G, Yu HQ. Biohydrogen production by mixed culture at various temperatures. Int J Hydrogen Energy 2006;31:780–5.
[35] Chaisri S, Boonsawang P, Prasertsan P, Chaiprapat S. Effect of organic loading rate on methane and volatile fatty acids production from anaerobic treatment of palm oil mill effluent in UASB and UFAF reactors. Songklanakarin J Sci Technol 2007;(Suppl. 2):311–23.
[36] Najafpour GD, Zinatizadeh AAL, Mohamed AR, Hasnain Isa M, Nasrollahzadeh H. High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor. Process Biochem 2006;41:370–9.
[37] Neves LCMD, Converti DNA, Penna TCV. Biogas production: new trends for alternative energy sources in rural and urban zones. Chem Eng Technol 2009;32:1147–53.
[38] Karakashev D, Batstone DJ, Angelidaki I. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl Environ Microbiol 2005;71:331–8.
[39] Shin SG, Han G, Lim J, Lee C, Hwang S. A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater. Water Res 2010;44:4838–49.
[40] Borja R, Banks C, Sanchez E. Anaerobic treatment of palm oil mill effluent in a two-stage up-flow anaerobic sludge blanket (UASB) system. J Biotechnol 1996;45:125–35.
[41] Kyazze G, Dinsdale R, Guwy AJ, Hawkes FR, Premier GC, Hawkes DL. Performance characteristics of a two-stage dark fermentative system producing hydrogen and methane continuously. Biotechnol Bioeng 2007;97:759–70.
[42] Ueno Y, Fukui H, Goto M. Operation of a two-stage fermentation process producing hydrogen and methane from organic waste. Environ Sci Technol 2007;41:1413–9.
[43] Vijayaraghavan K, Ahmad D. Biohydrogen generation from palm oil mill effluent using anaerobic contact filter. Int J Hydrogen Energy 2006;31:1284–91.
[44] Koutrouli EC, Kalfas H, Gavala HN, Skiadas IV, Stamatelatou K, Lyberatos G. Hydrogen and methane production through two-stage mesophilic anaerobic digestion of olive pulp. Bioresour Technol 2009;100:3718–23.
[45] Kongjan P, O-Thong S, Angelidaki I. Hydrogen and methane production from desugared molasses using a two-stage thermophilic anaerobic process. Eng Life Sci 2013;13:118–25.
[46] Yu H, Zhu Z, Hu W, Zhang H. Hydrogen production from rice winery wastewater in an up-flow anaerobic reactor by using mixed anaerobic granules. Int J Hydrogen Energy 2002;27:1359–65.